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## A MOLECULAR AND HAEMATOLOGICAL STUDY OF *THEILERIA EQUI* IN BALKAN DONKEYS

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Equine piroplasmosis in donkeys has been recognised as a serious problem of major economic importance. The present molecular study is the first investigation of the presence of *Theileria equi* and *Babesia caballi* in Balkan donkeys and of the possible haematological alterations related to it. A total of 70 apparently healthy donkeys from Serbia were included in this study. The overall prevalence of *T. equi* infection in donkeys tested with multiplex PCR was 50%. There was no *B. caballi*-positive sample. Infections in donkeys included in this study seem to be associated with decreased red blood cell count, haemoglobin concentration, haematocrit and platelet count, and with increased white blood cell count, mean corpuscular haemoglobin and mean corpuscular haemoglobin concentration. Altered haematological parameters in donkeys can lead to a decrease in working capacity and production performance. Further molecular research and long-term monitoring of equine piroplasmosis is needed in Serbia and throughout Europe.

**Key words:** Equine piroplasmosis, donkeys, PCR, haematological alterations, Serbia

The Balkan donkey is an endangered autochthonous breed confined to the Balkans and viewed as an unselected, unstructured population threatened by depopulation, inbreeding and stochastic traditional management (Stanisic et al., 2015). Reduction in the size of the population over the last decades came as a result of intensive agrarian production and modern farming trends. Due to additional socioeconomic changes in the rural areas, the current number of sexually mature donkeys in Serbia has been estimated at 250–300 individuals only (Stanisic et al., 2015). Over the past few years, interest in the welfare and diseases of donkeys has been constantly increasing due to the rediscovery of donkey milk and its different uses as well as due to the use of donkeys in sports activities, for rec-

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reational purposes, donkey-assisted therapy, as pack/draught animals and for meat production (Wise et al., 2013; Laus et al., 2015).

Equine piroplasmosis (EP) is one of the most important tick-borne disease in equids (horses, donkeys, mules and zebras) caused by the intraerythrocytic haemoprotozoan parasites *Theileria equi* and *Babesia caballi* (Mehlhorn and Schein, 1998; Kumar et al., 2009). EP has a worldwide distribution and is endemic in tropical, subtropical and some temperate regions (Rothschild, 2013). Piroplasmosis in donkeys has been recognised as a serious problem of major economic importance since the affected animals manifest loss of appetite and decreased working capacity. Donkeys usually show an asymptomatic form of the disease (chronic cases), with lower parasitaemia compared to infection in horses (Kumar et al., 2009). Acute cases of piroplasmosis in donkeys are characterised by fever, listlessness, depression, noticeable thirst, swelling of the eyelids, constipation, presence of a yellow mucus covering the faeces, yellowish coloration of the urine, and splenomegaly (Laus et al., 2015). Chronic cases are usually characterised by nonspecific clinical signs such as mild inappetence, poor work performance and weight loss (Kumar et al., 2009).

So far only a single study has been conducted in Serbia about equine piroplasmosis in horses (Davitkov et al., 2016), and there is no report on the presence or prevalence of these parasites in donkeys in this region. The present study is the first molecular investigation aimed to examine the presence of *T. equi* and *B. caballi* in Balkan donkeys in Serbia, and to investigate the possible haematological alterations related to it.

## Materials and methods

### *Donkeys, sampling procedures and haematology analyses*

The majority of the donkey population in Serbia is situated in the following locations: the Special Nature Reserve (SNR) 'Zasavica', the Stara planina Mt. region and in Kovilj village near Novi Sad (Fig. 1) (Stanisic et al., 2015). A total of 70 apparently healthy donkeys from these three locations were included in this study: 25 donkeys from SNR Zasavica, 25 donkeys from Stara planina Mt. and 20 donkeys from Kovilj village. Blood samples were collected by jugular venipuncture into sterile tubes with anticoagulant (EDTA) for polymerase chain reaction (PCR) and complete blood count (CBC). Stress was minimised by handling the animals with care before the collection of samples. The samples were maintained under cool conditions and immediately transferred to the laboratory. Haematological profiles were performed within 12 h following the blood sampling. CBC was determined with an automatic cell counter (Abacus Junior Vet, Diatron, Austria), using the pre-formatted software for analysing equine blood. The following parameters were included in the haematological analysis: white blood cells (WBC), lymphocytes (LYM), mid cells count (MID), granulo-

cytes (GRA), red blood cells (RBC), haemoglobin (HGB), hematocrit (HCT), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), mean corpuscular volume (MCV) and platelet count (PLT).

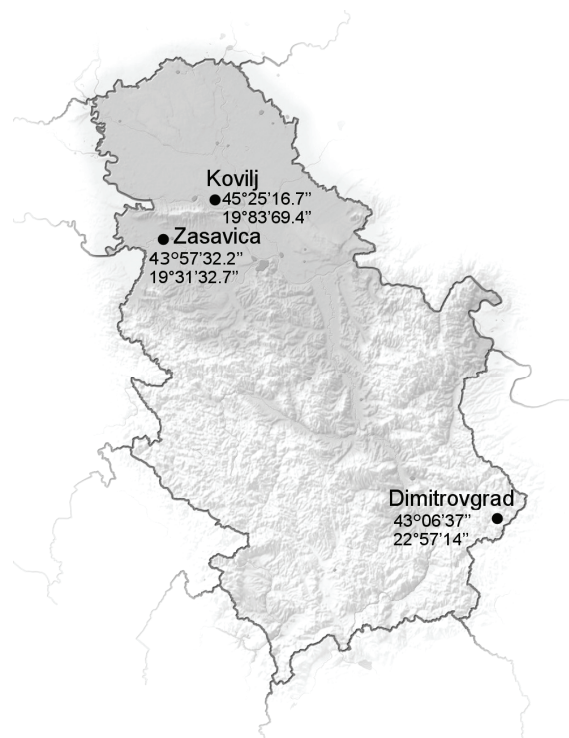


Fig. 1. Map of Serbia: the black dots indicate localities where samples were collected

#### DNA extraction and multiplex PCR

DNA was isolated from the blood samples using QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. Extracted DNA samples were stored at  $-20^{\circ}\text{C}$  until use. A multiplex PCR method for the simultaneous detection and differentiation of *T. equi* and *B. caballi* was employed with the following set of primers: Bec-UF2 as a universal forward primer and Cab-R and Equi-R as reverse primers specific for *B. caballi* and *T. equi*, respectively (Alhassan et al., 2005). This set of primers amplifies specific fragments of the 18S rRNA gene (392 bp for *T. equi*, 540 bp for *B. caballi*).

All PCRs were performed in a MultiGene Gradient Thermal Cycler (Labnet International Inc., Edison, NJ, USA) in 25- $\mu\text{L}$  volumes containing 12.5  $\mu\text{L}$  of KAPA2G Robust HotStart ReadyMix (Kapa Biosystems, Wilmington, Massachusetts, USA, PN KK7152), 1.25  $\mu\text{L}$  of each primer (10  $\mu\text{M}$ ) and 10  $\mu\text{L}$  (10 ng/ $\mu\text{L}$ )

DNA sample. The PCR parameters for amplification were: initial DNA denaturation of 3 min at 95 °C followed by 35 cycles of 15 s at 95 °C, 15 s at 58 °C and 15 s at 72 °C, and terminated with a final extension step at 72 °C for 8 min. Amplification products were separated on a 2% agarose gel stained with ethidium bromide and visualised under UV light.

#### *DNA sequencing*

To confirm the PCR results all positive PCR products were directly sequenced in two directions using the BigDye<sup>®</sup> Terminator method in an ABI 3730XL automatic DNA sequencer (Macrogen Europe, Amsterdam, The Netherlands). Sequence similarity analysis was performed using the BioEdit version 7.2.5 and Clustal W software. Sequence homology searches were made using the online version of BLAST software (available at <http://blast.ncbi.nlm.nih.gov>). Finally, the representative sequences were deposited into GenBank (accession numbers for *T. equi*: KY319140, KY319141, KY319142).

#### *Statistical analysis*

Haematological parameters were tested for normality by the Kolmogorov–Smirnov test and then analysed by Student's *t*-test or Mann–Whitney *U*-test for comparison between positive and negative animals. Statistical significance was assessed at the 0.05, 0.01 and 0.001 probability levels. Odds ratios with 95% confidence intervals were calculated according to Bland and Altman (2000). Statistical analysis of the results was done using software GraphPad Prism version 6.00 for Windows (GraphPad Software, San Diego, California, USA, [www.graphpad.com](http://www.graphpad.com)).

### **Results**

The prevalence of *T. equi* infection in the 70 donkeys tested with multiplex PCR was 50% (Fig. 2). Prevalence rates within herd are presented in Table 1. There was no *B. caballi*-positive sample or mixed infection.

Abnormal haematological alterations with respect to normal ranges were detected in 38 samples (54%). Thirty-five of these donkeys (92%) were found PCR positive for *T. equi*. Haematological alterations in PCR-positive samples included decreased RBC (35/35), decreased HCT (21/35), decreased HGB (14/35), increased MCH (11/35), increased MCHC (18/35), increased WBC (16/35), increased LYM (11/35), and decreased PLT (15/35). The chance (odds ratio) that haematocrit is lower than normal in donkeys positive for *T. equi* is 51 times higher (95% confidence interval of 5.61–301.40) compared with donkeys negative for *T. equi*. The statistical differences between PCR positive and negative animals are shown in Table 2.

**Table 1**Intra-herd and total prevalence of *Theileria equi* and *Babesia caballi* using molecular testing

Herd	Location	Number of donkeys	PCR prevalence of <i>T. equi</i>	PCR prevalence of <i>B. caballi</i>
1	Zasavica	25	64% (16/25)	0%
2	Stara planina	25	48% (12/25)	0%
3	Kovilj	20	35% (7/20)	0%
Total		70	50% (35/70)	0%

**Table 2**Statistical association of haematological parameters with PCR positivity for *T. equi*

Parameters	PCR-negative for <i>T. equi</i> Mean (SD)	PCR-positive for <i>T. equi</i> Mean (SD)	
WBC	12.30 (1.87)	14.23 (3.76)	P = 0.0083**
LYM	5.57 (1.64)	6.61 (2.88)	P = 0.054
MID	0.40 (0.35)	0.40 (0.41)	P = 0.97
GRA	6.16 (1.99)	7.23 (3.44)	P = 0.116
RBC	7.21 (0.46)	6.01 (0.63)	P = 0.0001***
HGB	124.3 (7.15)	112.7 (12.87)	P = 0.0001***
HCT	37.94 (2.45)	30.41 (4.73)	P = 0.0001***
MCV	53.60 (3.01)	50.49 (6.28)	P = 0.0101*
MCH	17.45 (0.89)	18.87 (2.18)	P = 0.0007***
MCHC	326.4 (8.41)	378.3 (56.32)	P = 0.0001***
PLT	238.2 (79.23)	159 (115.3)	P = 0.0013**

SD = standard deviation. Statistical significance: \*P &lt; 0.05; \*\*P &lt; 0.01; \*\*\*P &lt; 0.001

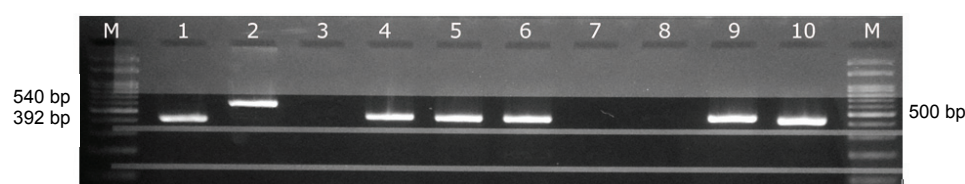


Fig. 2. Multiplex PCR detection of *Theileria equi* and *Babesia caballi*. M: 100-bp ladder DNA marker; lane 1: positive control for *T. equi*; lane 2: positive control for *B. caballi*; lane 3: negative control; lanes 4, 5, 6, 9 and 10: specimens infected with *T. equi*; lanes 7 and 8: negative specimens. The sizes of the positive bands are indicated on the left

Results of the sequence analysis demonstrated 100% correspondence to the species diagnosed by multiplex PCR. Sequences of the amplified DNA fragments were identical (391/391 bp) with the 18S rRNA gene sequences of *T. equi* deposited in GenBank (GenBank accession no.: KR351291.1, KF559357.1 and JQ657703.1).

## Discussion

To the authors' knowledge, this is the first molecular survey of *T. equi* in donkeys from Serbia. The results show that *T. equi* is widespread in this region and can lead to evident haematological alterations.

All donkeys were asymptomatic at the time of blood sampling, which is in accordance with literature reports that EP infections in donkeys are in most cases subclinical or chronic, with non-specific signs (Kumar et al., 2009). Chronically infected animals may act as parasite carriers for tick transmission and facilitate the spread of this potentially severe disease in horses.

Molecular methods have previously been used to detect the DNA of *T. equi* and *B. caballi* in horse blood samples from Serbia (Davitkov et al., 2016). In this study the prevalence values based on molecular techniques differed depending on the sampling location. Although the differences were not statistically significant, the prevalence of *T. equi* was the highest in Zasavica (64%). Such variations in prevalence between different sampling sites might be related to the presence of competent tick vectors, host activity or different climatic factors (Heim et al., 2007; Kouam et al., 2010; Grandi et al., 2011). The overall prevalence of *T. equi*-infected donkeys in our study was higher than that found in donkeys in Brazil (Machado et al., 2011) but lower than that reported from Iran and Italy (Abedi et al., 2015; Laus et al., 2015). In the present study, *B. caballi* infection was not detected in the blood samples of donkeys. This may be associated with the fact that infected equines completely eliminate *B. caballi* from their circulation after 1–4 years, while *T. equi* remains a life-long infection (de Waal and Van Heerden, 1994). Therefore, failure to detect *B. caballi* by PCR is perhaps due to parasite clearance from the blood by the host or the decrease of its presence to a level beyond the detection sensitivity of the molecular method used (Salim et al., 2008).

In agreement with our results, most epidemiological studies on EP showed a higher prevalence of *T. equi* compared with *B. caballi* infections in donkeys (Laus et al., 2015) as well as in horses (Bashiruddin et al., 1999; Criado-Fornelio et al., 2004; Nagore et al., 2004; Acici et al., 2008; Kouam et al., 2010; Ros-García et al., 2013; Davitkov et al., 2016). The higher prevalence of equine theileriosis can be explained by the higher parasitaemia in the infected animals, the longer persistence of *T. equi* than *B. caballi* after infection, and the transplacental transmission of *T. equi* (de Waal and van Heerden, 1994; Allsopp et al., 2007; Georges et al., 2011).

Subclinical natural infections in donkeys included in this study seem to be associated with decreased RBC, HGB, HCT and PLT and with increased WBC, MCH and MCHC. These results correspond well to the abnormalities reported in a recent study in Italy (Laus et al., 2015). The main haematological alterations in *T. equi* infections described in the present study were anaemia, leukocytosis, and

thrombocytopenia. All these clinicopathological findings suggest the presence of a direct and immune-mediated pathogenic activity of the parasites. All infected animals had decreased RBC.

The present work showed that *T. equi* is prevalent among Balkan donkeys in Serbia. The identified haematological alterations in donkeys can lead to reduced working capacity and production performance. Veterinary examinations and health status monitoring are very important for preserving the Balkan donkey as an animal genetic resource. Also, infected donkeys act as parasite carriers and facilitate the spread of equine piroplasmosis, which has a major impact on international trade and on the horse industry. Further molecular research and long-term monitoring of equine piroplasmosis is needed in Serbia and throughout Europe.

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